Role of IL-12 in T cell activation in old mice

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirement for Graduation *with Distinction* in Microbiology in the Undergraduate College of Biological Sciences of the Ohio State University

By Craig Davis

The Ohio State University
February 2008

Project Advisors: Joanne Turner, PhD, Assistant Professor of Internal Medicine with a joint appointment in the Department of Molecular Virology, Immunology, and Medical Genetics at The Ohio State University Medical Center

Bridget Vesosky, PhD, Senior Research Associate in the Center for Microbial Interface Biology at The Ohio State University Medical Center

Background and Abstract

The cytokine interleukin-12 (IL-12) has potent biological activities and is particularly important for the synergistic activation of $CD4^+$ and $CD8^+$ T cells to secrete IFN-γ during an antigen specific exposure. Additionally, IL-12 can stimulate cells of the innate immune system (natural killer cells) directly to secrete IFN-γ. In old mice it has been shown that IL-12 can stimulate $CD8^+$ T cells to secrete IFN-γ in an antigen-independent manner, demonstrating an alternative T cell activation pathway in old age. This project seeks to dissect the mechanism of $CD8^+$ T cell activation in more detail. Biologically active IL-12 exists as a heterodimer comprised of two peptide chains, p40 and p35. In this form IL-12 (p70) can interact with the IL-12 receptor β1 and β2 chains and signal the receptive cells to produce IFN-γ. The single peptide p40 can also exist as a monomer (p40) or a homodimer (p40/p40, designated p80). It has been shown under various conditions that p80 can stimulate or inhibit T cells. This project determined whether the stimulatory effect of IL-12 on $CD8^+$ T cells from old mice to produce IFN-γ is mediated by IL-12p70, p80 homodimer, or p40 monomer. Additionally, we determined whether IL-23, a close relative of IL-12 consisting of p40 and p19, can stimulate $CD8^+$ T cells directly. Using combinations of p40 monomer, p40 homodimer, IL-12, or IL-23 we stimulated T cells to secrete IFN-γ and measured cytokine production by enzyme linked immuno-sorbent assay (ELISA). Dose response curves and time course were performed to optimize the assays. Once the optimum assay conditions were ascertained, the stimulatory component of IL-12 was identified. Additional studies determined whether p40 or p80 can inhibit the biological action of IL-12. Furthermore, an additional

synergistic cytokine, IL-18 was added into the assay conditions to determine whether the stimulatory capacity of IL-12 and its family members can be boosted. Finally, to conclusively demonstrate that $CD8^+$ T cells from old mice are directly stimulated to produce IFN-γ by an IL-12 family member, cell purification techniques were implemented.

Introduction

Tuberculosis Prevalence in the World – Tuberculosis (TB) is a contagious disease primarily spread by way of the respiratory route (1). TB is caused by the slow-growing acid-fast bacillus *Mycobacterium tuberculosis* (*M.tb*) (2). Though TB can affect many different organs, pulmonary TB is most common (5). It is estimated that one-third of the world's population is currently infected with *M.tb*, but infection does not always lead to the active form of TB (3). In a vast majority of people, *M.tb* will remain dormant for several years without any symptoms (latent TB) yet still having the potential to reactivate. If left untreated or undetected, it is estimated that each person with active TB will spread *M.tb.* to 10-15 more people each year (3). Reactivation of TB is often seen in patients who are immune-compromised, especially those with HIV (4). With HIV as a major contributor to the increase of incidence of TB, Africa had both the highest number of deaths and highest mortality per capita due to TB (3). TB also affects many other parts of the world as well. Southeast Asia represents one-third of TB cases reported globally (3). Although the incidence throughout the world is on decline, with population growth the number of new cases is constantly increasing. Thus TB remains one of the most prevalent infectious diseases in the world resulting in 2-3 million deaths per year and 8-10 million newly reported cases each year(3).

Increased Susceptibility in the Elderly - It is well known that the elderly are more susceptible to infectious diseases including pneumonia, influenza, TB and that increased susceptibility is associated with a decrease in immune response. Pneumonia is the fourth leading cause of death among those over the age of 65 (6). In addition to pneumonia as a common disease in the elderly, those over the age of 65 also have a four times greater risk of developing TB when compared to those under 65. When living within a nursing home the chance of developing TB increases up to three fold more (25). Additionally, 75% of all elderly TB cases are respiratory and the majority of the cases seen in the elderly are caused by reactivation of latent TB (6).

Early, transient resistance to *M. tb* in old mice –Like elderly humans, old mice are also more susceptible to *M.tb* and succumb to disease faster than young mice. Despite this, it has been shown that old mice display an early transient resistance to infection with *M.tb* (7). This early transient resistance can be associated with a few major factors. In old mice, there is an increased number of $CD8^+$ T cells in the lungs capable of antigen independent IFN-γ production (8). IL-12 and 18 have been shown to stimulate T cells in an antigen independent manner (11). These cytokines are elevated in the lungs of old mice infected with *M.tb* (9). At 12 days post infection, lungs from old mice contained at least two times more IL-12p40 mRNA than the lungs of young mice (9). Furthermore it was shown that there was more IL-18 protein in the lungs of old mice 12 days post infection (9). It has also been shown that there is an increase in the level of Th1 cytokine receptor expression in old mice. Th1 cytokine mediated immunity is critical to the early

transient resistance of old mice to *M.tb* infection (9). The mechanism by which IL-12 stimulates CD8 T cells from old mice to secrete IFN-γ will be the focus of this study.

IL-12

Structure - Interleukin-12, a heterodimeric protein, is composed of two subunits, 40 kDa (p40) and 35 kDa (p35). Joined by a disulfide bridge, the p40 and p35 subunits are composed of 306 and 197 amino acids, respectively (11). The p40 gene is over 14 kb long and the p35 gene is over 8 kb long (13). Each is located on a separate chromosome, and the promoter region for each gene is different which accounts for the difference in expression regulation (13). Both subunits contain cysteine residues important in posttranslational modifications. The p40 subunit includes 10 cysteine residues whereas the p35 subunit includes 7 cysteine residues (11). The IL-12 subunits undergo several modifications including posttranslational glycosylation (12). More specifically, the p35 subunit undergoes modification with sialic acid added to N-linked oligosaccharides (12). The posttranslational modifications of p35 distinguishes the immature intracellular and mature secreted heterodimer of IL-12 (12).

Production - IL-12 is produced by macrophages, monocytes, dendritic cells, neutrophils and B Cells (11,14,15). The p40 gene is expressed by cells that are able to form IL-12 while isolated p35 protein has not been detected (12). The p40 gene is regulated at transcription and has transcription factors including NF-kb, IRF-1, c-Rel, interferon consensus binding protein and Ets family members (16). The p35 gene is regulated both transciptionally and translationally (12,16). After transcription the p35 mRNA contains

an inhibitory ATG site that stops translation. Upon stimulation this site is deleted and translation proceeds.

In response to microbial products such as LPS, lipoteichoic acid and peptidoglycan, IL-12p40 and IL-12p70 are highly induced (17). Macrophages and dendritic cells are thought to be the primary producers of IL-12p70.

Receptor - The IL-12 receptor (IL-12R) is mainly expressed on activated T cells and Natural Killer (NK) cells. The receptor for IL-12 is composed of two subunits, β1 and β2 (18). Both subunits are Type 1 transmembrane glycoproteins (16). IL12-Rβ1 has a molecular size of 100 kDa while IL-12Rβ2 has a molecular size of 130 kDa (16). Both subunits are necessary for high affinity binding (16). The p40 subunit interacts with the β1 subunit of the receptor and provides the binding energy while p35 interacts with the β2 subunit of the IL-12R (19).

Signaling Pathway of IL-12 and IL-12R - The β2 subunit signals through intracellular tyrosine residues which are phosphorylated by Janus kinases (JAK)(18,20) . This phosporylation of tyrosines creates a docking station for SH-2 domain of the STAT4 protein and JAK catalyzes the phosphorylation of the tyrosine in STAT4 (20) (Figure 1). In the murine model, tyrosine residues 757, 804, 811 are each independently capable of phosphorylating STAT4 tyrosine in response to IL-12 (21). Phosphorylated STAT4 (pSTAT4) then dimerizes with another pSTAT4. Once phosphorylated, dimerized pSTAT4 translocates to the nucleus where it is involved in IFN-γ production, though the exact mechanism is not known.

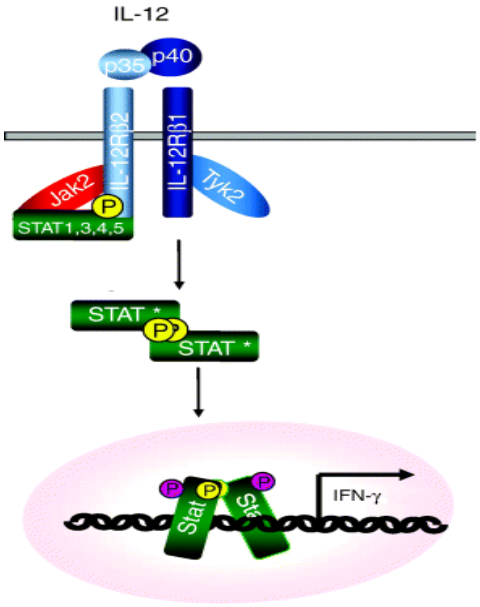

Figure 1. Image of IL-12 JAK/STAT Signaling Pathway.

Picture Modified from Wendy T. Watford, Bruce D. Hissong, Jay H. Bream, Yuka Kanno, Linda Muul, John J. O'Shea (2004) Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4 Immunological Reviews 202 (1), 139–156

IL-18

IL-18 is structurally related to the members of the IL-1 family. IL-18 is synthesized as an inactive precursor and upon cleavage by intracellular cysteine protease (ICE, caspase 1), becomes biologically active (24). IL-18 binds to the IL-18R receptor to transduce its signal. The receptor has two subunits, IL-1 Receptor related protein (IL-1Rrp) and IL-18R (24). IL-18 signals through a complex signaling cascade, ultimately resulting in the translocation of NFkB to the nucleus (23). IL-18 is produced by monocytes and macrophages (24).

IL-12 and IL-18 effect on T cells

Synergism – IL-12 and IL-18 act synergistically to increase IFN-γ production by T cells (22). Studies have shown IL-18-deficient mice produce a decreased amount of IFN-γ as compared to wild-type mice. The levels of IL-12 in IL-18 deficient mice were shown to be at the same levels as wild type mice (24). IFN-γ production in IL-18-deficient mice was lower indicating that the synergism of IL-12 and IL-18 is not in the production of

either cytokine but rather they act synergistically on the T cells that produce IFN-γ (24). It has also been shown that T cells stimulated with IL-12 showed and increased expression of IL-18R, thus providing a possible mechanism of synergism (23) (Figure 2).

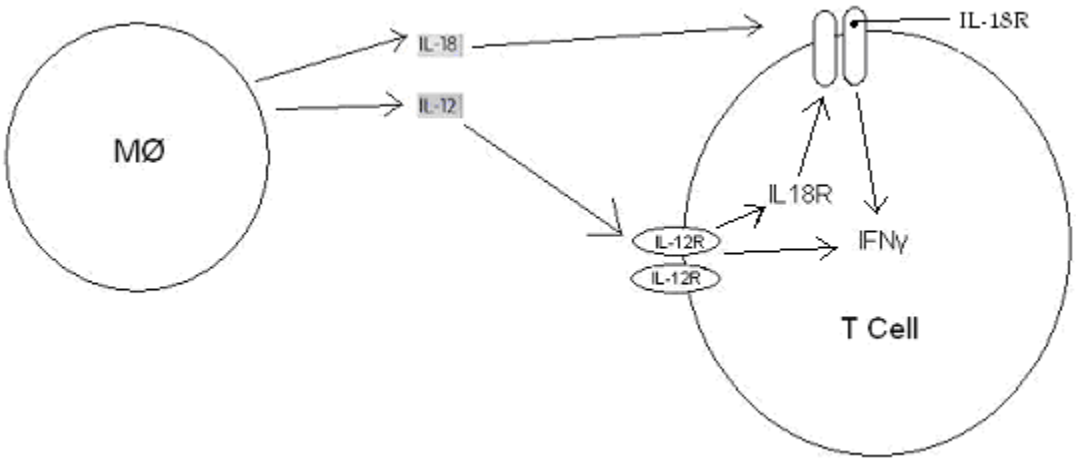

Figure 2. Possible mechanism behind IL-12/IL-18 synergism. Macrophage (MØ)

Upon infection with *M.tb,* old mice produce an antigen independent response that increases resistance early after infection. This early resistance was shown to be associated with an increased expression of Th1 cytokine receptors on $CD8^+$ T cells as the age of the mouse increased as well as an increased $CD8^+$ T cell population in the lungs of old mice infected with *M.tb* (9). This study seeks to investigate how two of the Th1 cytokines (IL-12 and IL-18) activate $CD8^+$ T cells *in vitro.* This study also investigates the various effects of IL-12p40, IL-12p40/p40 and IL-23, a close relative of IL-12. It is essential to identify the Th1 cytokine associated with the early transient resistance to *M.tb* in old mice. Exploring the mechanism behind this early transient resistance and enhancing this resistance can lead to new therapeutic strategies in the elderly that are more effective than

current strategies. **We hypothesize that IL-12p70 is the only p40 containing cytokine that can stimulate $CD8^+$ T cells from old mice to secrete IFN-γ, and also requires to IL-18 to fully stimulate IFN-γ production.** Stimulation of whole spleen cultures will be done to optimize the assay then $CD8^+$ T cell purification techniques will be done to directly test the effects of IL-12 and IL-23, p40 containing cytokines. The spleens will be used as the source of $CD8^+$ T cells since there is a larger population of $CD8^+$ T cells in comparison to the lungs

Methods and Materials

Tissue Culture Media– Contains 1 bottle 500 ml Dulbecco's Modification of Eagle's Media (Cellgro/Fisher MT15-017-CV), 10 ml minimal essential amino acids (Sigma M7145), 5 ml HEPES buffer (Sigma H0887), 5 ml L-glutamine (Sigma G7513), 5 ml Penicillin/Streptomycin (Sigma P0781), 660 µl 2-mercaptoethanol (50mM), 45 ml heat inactivated fetal bovine serum (Atlas F-0500-D, Lot A20530B)

Gey's Solution – 4.15g NH_4Cl (8mM), 0.5g $KHCO_3$ (5mM), 500 ml bottled LPS free H_20. It is then sterile filtered.

Single Cell Suspension

Whole spleens from young (2-3 months) and old (17-18 months) C57BL/6 and BALB/c mice were gently passed through a 70 µm cell strainer (BD Falcon) to form a single cell suspension. Cell suspensions were then equalized to 5 mL using tissue culture media (TCM). The cell suspension was centrifuged for 7 min at 1046 x g at 4° C. The supernatant was poured off resulting in a red color cell pellet. The red pellet was

resuspended and 2.5 mL Gey's Solution was added for 3 minutes to lyse the red blood cells. After 3 minutes, the reaction was stopped with 5 mL TCM. The cell suspension was centrifuged for 7 min at 1200 rpm and 4° C. After centrifugation, the supernatant was poured off and the cell pellet was resuspended in 5 mL of TCM. 10 uL of cell suspension was added 90 uL of Trypan blue. The cells were counted using a hematocytometer and adjusted to a concentration of 1x 10^6 cells/mL.

Cell Purification

Single cell suspensions from spleens were centrifuged at 1046 x g for 7 minutes at 4 ^oC, supernatants removed and 50 uL of anti-mouse CD8a particles were added for every 10^7 cells. The suspension was incubated for 30 minutes at 4^oC. 1mL TCM was added and the suspension was placed on the BD ^{TM} Imagnet for 7 minutes. Supernatant was saved for analysis by flow cytometry for evaluation. The cells were resuspended in 1 ml TCM and placed on the magnet for 4 minutes. This process was repeated twice more. A final sample of purified CD8 T cells was taken to evaluate cell purity (Purity >85%). Purified cells were adjusted at 1x10^6 cells/ml.

In Vitro Cell Culture

Cells were cultured at 1x10^6/ml in a 96- well plate in a humidified incubator at 37^oC and 5 % CO_2 . See Table #1 for the concentration of each cytokine in cell culture. These concentrations were based upon concentration optimization experiments. In the time course assay, cell samples were taken at 24, 48, 72, and 96 hours. All other *in vitro* cell cultures were incubated for 48 hours based upon optimization. After 48 hours, cultures were frozen at –20°C for analysis by ELISA.

Table 1. Information on Cytokines used

Cytokine	Company	Catalog #	Lot #	Concentration
IL-12p70	Peprotech	210-12	1205S97 L055	5 ng/ml
IL-12p40	Peprotech	210-11	0704S305 G294	20 ng/ml
IL-12p40/p40	R&D Systems	499-ML-025/CF	RH014051	20 ng/ml
IL-23	R&D Systems	1887-ML-010/CF	MLE 036031	20 ng/ml
IL-18	R&D Systems	B004-5	N/A	20 ng/ml

Analysis for IFN-γ by ELISA

Cell culture supernatants were analyzed for IFN-γ by ELISA. 96 well Microtest™ ELISA Plates (BD Falcon) were coated with purified rat anti-mouse IFN-γ (BD Pharmingen) in 0.1 M Sodium Bicarbonate buffer, pH 8.2. Plates were incubated for 24 hours at 4°C. The wells were blocked with 200 uL TCM (containing 10% Fetal Bovine Serum). 100 uL of each sample or standard (Concentration 25 ng/ml-0.39 ng/ml) was dispensed in duplicate or triplicate and incubated for 1 hour. Wells were washed with PBS containing 0.5% Tween 20 (PBS-T). 100 uL secondary anti-IFN-γ biotinylated antibody was added at 1ug/ml and allowed to sit at room temperature for 1 hour. The plate was washed followed by adding dilute (1:4000) Streptavidin Horseradish Peroxidase (Zymed, 43-4323). The Streptavidin Horseradish Peroxidase was allowed to sit at room temperature for 30 minutes followed by a wash with PBS-T. 3,3',5,5,-tetramethylbenzidine substrate (Invitrogen) was added. The reaction was stopped with

100 µl H_2SO_4 and read at 450 nm. Standard curve was generated and the concentration of IFN-γ (ng/ml) was determined for each sample.

Results

Cytokine Concentration Optimization

In order to determine if, and at what concentration IL-12p70, IL-12p40, IL-12p40p40 or IL-23 stimulate T cells, cell cultures of 100 µL were prepared from the spleens of old and young mice. The cytokines were titrated in 1:2 dilutions from 20 ng/mL through 1.25 ng/mL for 24 hours. Ovalbumin was used as a negative control. IFN-γ was measured by ELISA. Data analyzed showed that IL-12p70, IL-12p40, IL-12p40p40 and IL-23 showed little or no IFN-γ production above non-stimulated cells (Data not shown). Because IL-18 can synergize with IL-12, cell cultures from old (Figure 3) and young (Figure 4) were stimulated with IL-12p70, IL-12p40, IL-12p40p40 or IL-23 and prepared with IL-18 at 20 ng/ml per well. Cells were also incubated with IL-18 alone to account for background production of IFN-γ by IL-18. Cell cultures containing IL-12p40, IL-12p40p40, and IL-23 along with IL-18 added to each well showed no IFN-γ production above non-stimulated cells. The cell cultures containing IL-12p70 and IL-18 produced IFN-γ. ELISA results showed a dose dependent IFN-γ response to IL-12p70/IL-18 that reached a plateau at 2.5 ng/mL of IL-12p70.

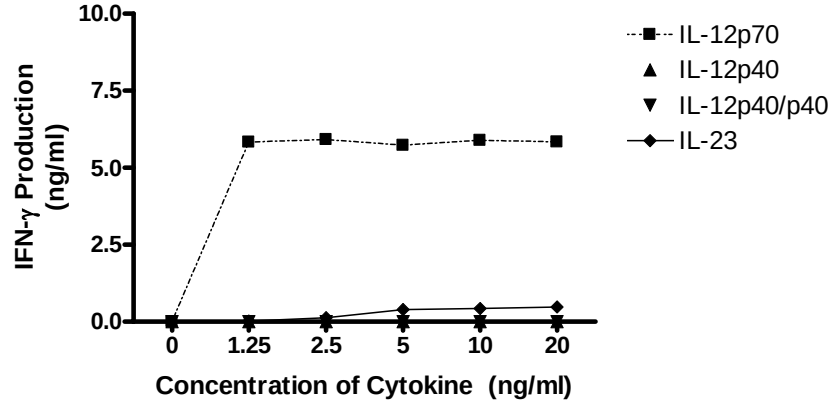

Figure 3. Optimization of cytokine concentrations using spleens from old mice. Cytokine-induced production of IFN–γ by splenocytes. Splenocytes of 17-month-old mice were cultured for 24 h with media and etiher IL-12p70, IL-12p40, IL-12p40/p40 or IL-23. All cytokines were in 1:2 dilutions from 20 ng/ml to 1.25 ng/ml. IFN-γ production was analyzed using ELISA. Data is representative of two independent experiments.

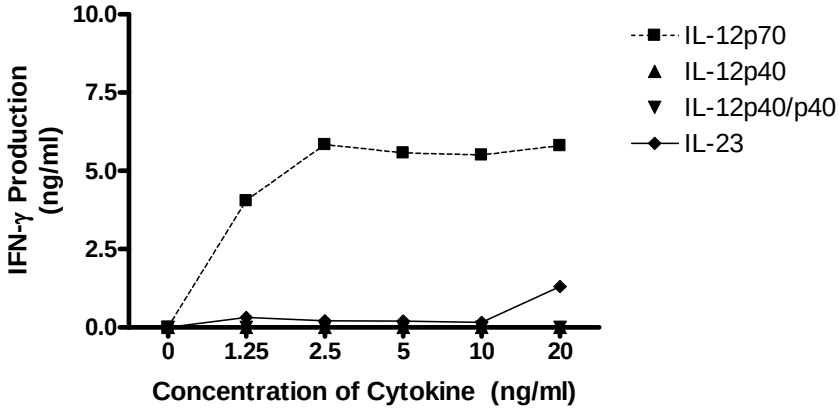

Figure 4. Optimization of cytokine concentrations using spleen cells from young mice. Cytokine-induced production of IFN-γ by splenocytes. Splenocytes of 2-month-old mice were cultured for 24 h with media and etiher IL-12p70, IL-12p40, IL-12p40/p40 or IL-23. All cytokines were in 1:2 dilutions from 20 ng/ml to 1.25 ng/ml. IFN-γ production was analyzed using ELISA. Data is representative of two independent experiments.

The optimal concentration of IL-12p 70 was determined to be 5 ng/mL since it was on the plateau of both young and mice. Because no signal was observed for IL-12p40, IL-12p40p40 and IL-23 these were subsequently used at 20 ng/ml.

Optimization of Incubation Time

An experimental time course was analyzed at 24, 48, 72 and 96 hours. The time course was performed to determine when the peak of IFN-γ production occurred. Cell cultures were prepared from single cell spleen suspensions. IL-12p70 was added to the cultures at 5 ng/mL and IL-12p40, IL-12p40p40 and IL-23 were cultured at 20ng/mL. The IL-12p70 cell cultures contained IL-18. For this experiment the IL-12p40, IL-12p40p40 and IL-23 cell cultures did not contain IL-18. Results for young and old mice were calculated from a total of three mice in independent experiments. In cell cultures from old mice containing IL-12p70 and IL-18 produced abundant IFN-γ within the first 48 hours (Figure 5). This increase in IFN-γ production was slowed by 50% between 48 and 96 hours. Cells from young mice cultured with IL-12p70/IL-18 produced slightly less IFN-γ with similar kinetics as old mice. In old mice IFN-γ production in response to IL-12p40 peaked at 96 hours (Figure 6). IL-23 stimulated IFN-γ production rising steadily between 24-96 hours (Figure 6). This IFN-γ production peaked at 96 hours with an IFN-γ production of 0.5 ng/mL. Cell cultures from old mice did not produce IFN-γ in response to IL-12p40/p40 (Figure 6). Cells cultured from the spleens of young mice did not produce IFN-γ in response to IL-12p40, IL-12p40/p40 and IL-23(Figure 7). From these data we concluded that 48 hr was optimum time point for analysis.

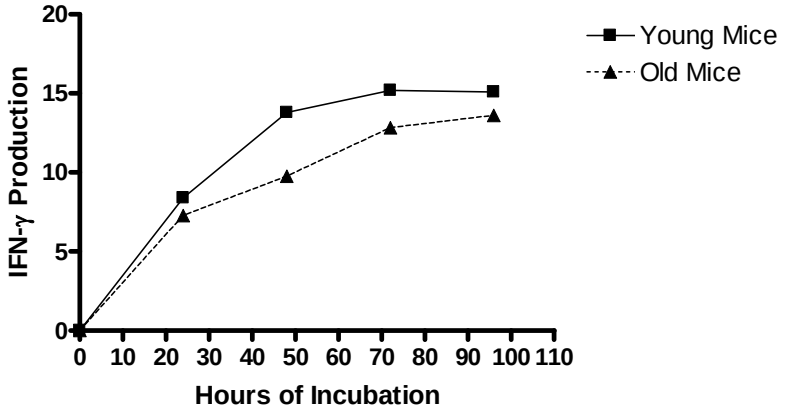

Figure 5. Optimization of incubation time using IL-12p70 in both young and old mice. IL-12p70/IL-18-induced production of IFN–γ by splenocytes. Spleens cells of 2-3 and 17-18 month old mice were cultured with IL-12p70 and IL-18. IL-12p70 was cultured at 5 ng/ml. Cell culture samples were taken at 24, 48, 72 and 96 hours. IFN-γ production was analyzed using ELISA.

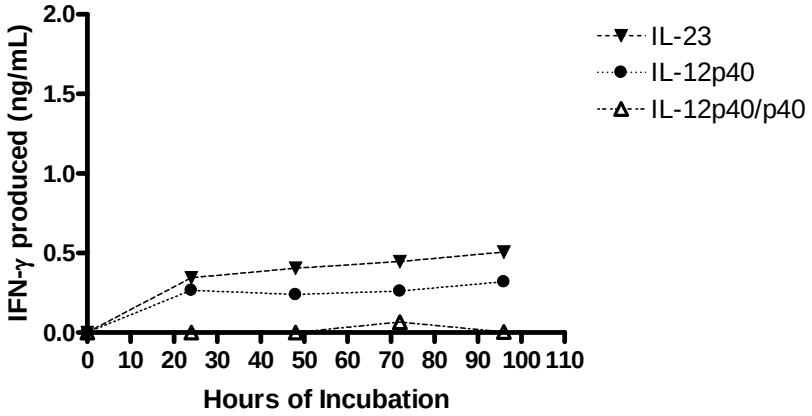

Figure 6. Optimization of incubation time using spleen cells from old mice, Cytokine-induced production of IFN-γ by splenocytes. Spleens cells of 17-18 month old mice were cultured with IL-12p40, IL-12p40/p40 and IL-23. All cytokines were cultured at 20 ng/ml. Cell culture samples were taken at 24, 48, 72 and 96 hours. IFN-γ production was analyzed using ELISA

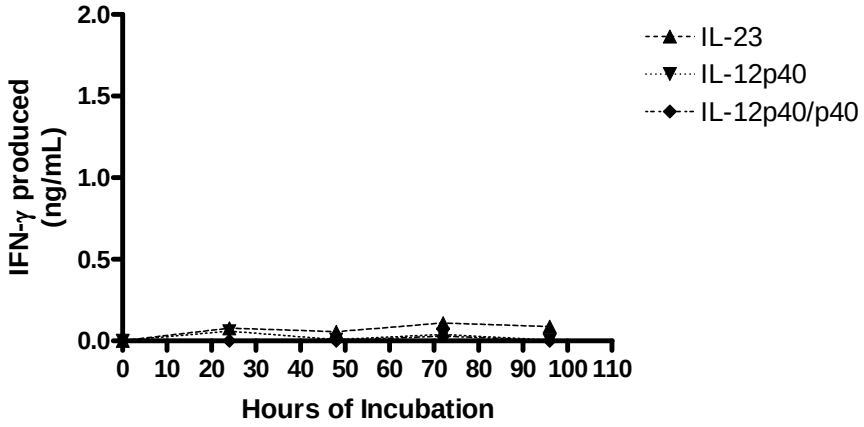

Figure 7. Optimization of incubation time using spleen cells from young mice, Cytokine-induced production of IFN-γ by splenocytes. Spleens cells of 2-3 month old mice were cultured with IL-12p40, IL-12p40/p40 and IL-23. All cytokines were cultured at 20 ng/ml. Cell culture samples were taken at 24, 48, 72 and 96 hours. IFN-γ production was analyzed using ELISA

Though both young and old cells showed small amounts or no IFN-γ stimulation with IL-12p40, IL-12p40p40 and IL-23 after 48 hours, the IFN-γ production levels were not enough to conclude that they are the major stimulatory factors for IFN-γ production. The timepoint chosen for incubation was based upon the findings for optimal stimulation with IL-12p70 in young and old mice. For both young and old mice the 48 hour timepoint was optimal IFN-γ production.

From the concentration optimization, 5 ng/ml was optimal for IL-12p70 for both young and old mice. 20 ng/ml was determined to be the optimal concentration for IL-12p40, IL-12p40/p40 and IL-23 in both young and old mice. 48 hours was shown to be the optimal incubation time for old and young mice. Because we saw minimal IFN-γ production in the IL-12p40, IL-12p40/p40 and IL-23 cultures, it was concluded that stimulation of spleen cells to secrete IFN-γ was specific to IL-12p70. This however will need to be verified using purified $CD8^+$ cells.

Inhibition of IFN γ secretion by IL-12p40/p40

IL-23, IL12p40 and IL-12p40/p40 can bind the IL-12R and therefore we determined whether these cytokines could interfere with the stimulatory activity of IL-12p70. Cells were cultured with IL-12p70 (5 ng/mL) in the absence or presence of IL-23, IL-12p40 or IL-12p40/p40. IL-12p40, IL-12p40/p40 and IL-23 were titrated in 1:2 dilutions from 20 ng/ml to 1.25 ng/ml and cultured with IL-12p70 and IL-18. In spleen cells of old mice, IFN-γ production in the presence of IL-12p70/IL-18 was not altered by IL-23 (Figure 8). Approximately 20% less IFN-γ was produced in the presence of IL-12p40 (Figure 8). In contrast, inclusion of IL-12p40/p40 decreased IFN-γ production (Figure 8).

In young mice, results were analogous (Figure 9). Cells cultured with IL-23 showed no change in IFN γ production. IL-12p40 showed a small amount of IFN-γ production reduction. Cells cultures with IL-12p40/p40 produced less IFN-γ, suggesting that IL-12p40 can inhibit but it is most effective when it is in homodimeric form.

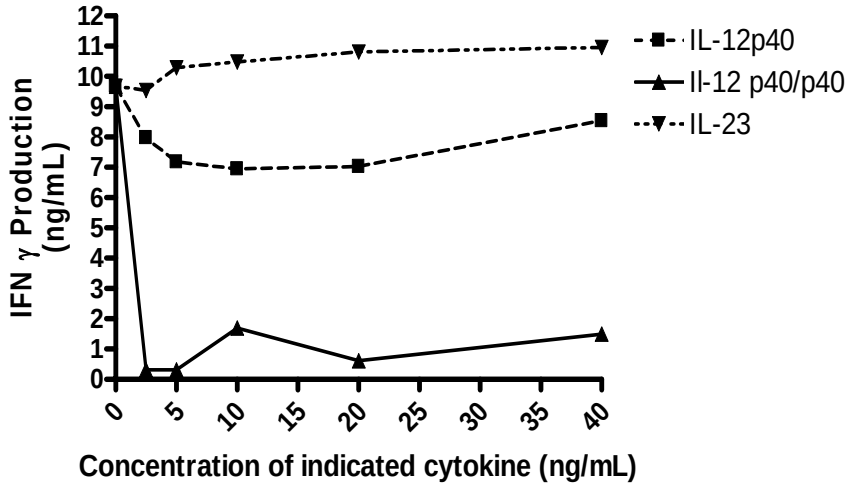

Figure 8. Inhibition of IFNγ production by spleen cells of old mice. Splenocytes of 17 month-old mice were cultured for 48 h with media, IL-12p70 and IL-18 in the absence or presence of Il-12p40, IL-12p40/p40, and IL-23. IFN-γ production was analyzed using ELISA. Data is representative of two independent experiments

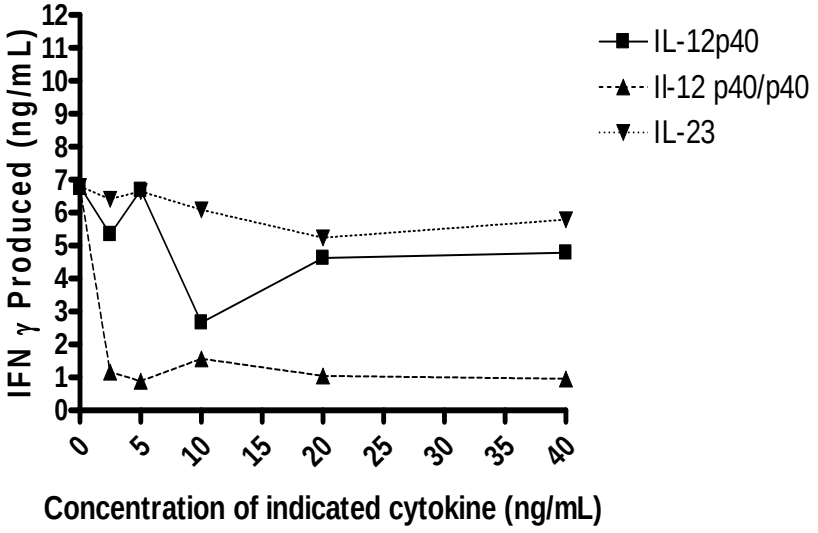

Figure 9. Inhibition of IFN-γ production by spleen cells of young mice. Splenocytes of 2 month-old mice were cultured for 48 h with media, IL-12p70 and IL-18 in the absence or presence of Il-12p40, IL-12p40/p40, and IL-23. IFN-γ production was analyzed using ELISA. Data is representative of two independent experiments

The inhibitory properties of IL-p40/p40 were evaluated in an independent experiment that compared results from young and old mice (Figure 10). Here, a dose response for inhibition was observed. When the inhibitory effect of IL-12p40/p40 was compared directly between young and old mice the dose response was relatively similar. A trend for decreased inhibition in old was evident. However, this will need to be verified with additional experiments.

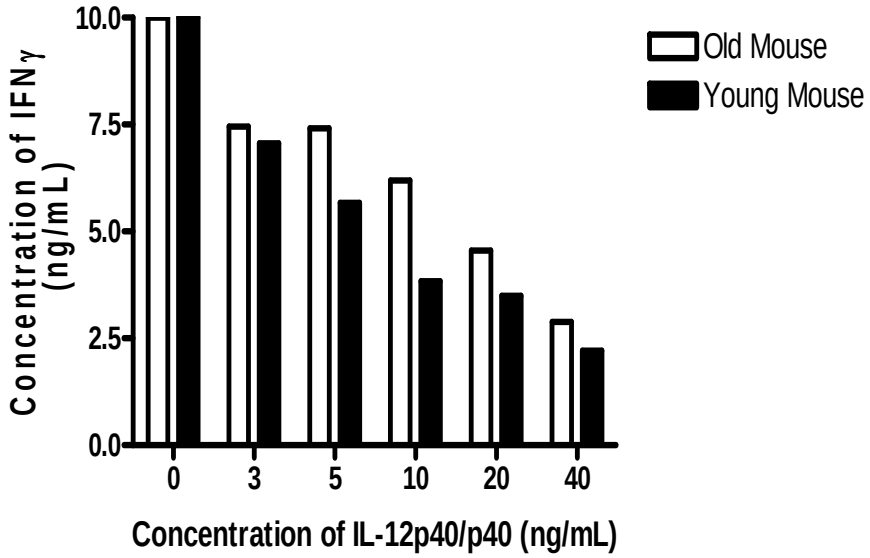

Figure 10. Dose dependent inhibition of IFN-γ production by IL-12p40/p40 in spleen cells of young and old mice. Splenocytes of 2-3 and 17-18 month-old mice were cultured for 48 h with media, IL-12p70 and IL-18 in the absence of presence of IL-12p40/p40. IFN-γ production was analyzed using ELISA. Data is representative of two independent experiments

IFN γ Production using $CD8^{\pm}$ T cells

Because other cell types produce IFN-γ,CD 8^{+} T cells were purified from the spleens of both young and old mice. It was therefore imperative that $CD8^{+}$ T cells were isolated for more specific analysis. $CD8^{+}$ T cells were incubated for 48 hours with IL-12p70, IL-12p40, IL-12p40p40 or IL-23. IL-18 was included in all cell cultures.

$CD8^{+}$ T cells from young mice did not respond to IL-12p40, IL-12p40p40, or IL-23 (Figure 11). $CD8^{+}$ T cells from young mice produced a large amount of IFN-γ upon stimulation with IL-12p70 and IL-18.

$CD8^{+}$ T cells from old mice (Figure 11) also showed no ability to respond to IL-12p40, IL-12p40p40 or IL-23. $CD8^{+}$ T cells from old mice produced a large amount IFN-γ when stimulated with IL-12p70.

These data verify that IL-12p70 is the cytokine that stimulates purified $CD8^{+}$ T cells to secrete IFN-γ.

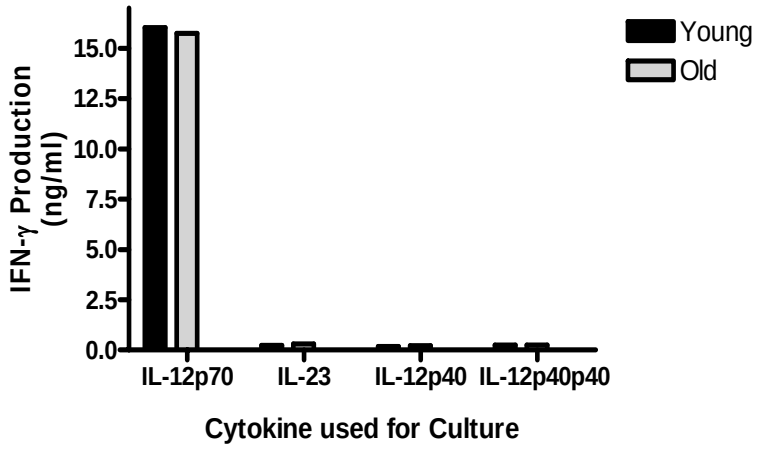

Figure 11. Cytokine-induced production of IFN-γ by $CD8^{+}$ T cells from the spleen of young and old mice. Purified $CD8^{+}$ T cells from the spleens of 2-3 and 17-18 month-old mice were cultured for 48 h with media and either IL-12p70, IL-12p40, IL-12p40/p40 or IL-23. IL-12p70 was cultured at 5 ng/ml. IL-23, IL-12p40 and IL-12 p40/p40 were cultured at 20 ng/ml. All cultures included IL-18. IFN-γ production was analyzed using ELISA. Data is representative of two independent experiments.

Inhibition of IFN-γ Production using purified $CD8^{\pm}$ T cells

To determine the effects of IL-12p40/40 on IL-12p70 induced IFN-γ production of purified $CD8^+$ T cells, $CD8^+$ T cells were purified from the spleens of old and young mice and cultured with IL-12p70/IL-18 in the absence or presence of IL-12p40/p40. IL-12p40/p40 was titrated in 1:2 dilutions from 0.0048 ng/mL – 40 ng/mL (Figure 12).

IL-12p40/p40 inhibited IFN-γ production in $CD8^+$ T cell cultures from young and old mice in a dose dependent manner. IFN-γ production was completely inhibited at 10 ng/ml or higher.

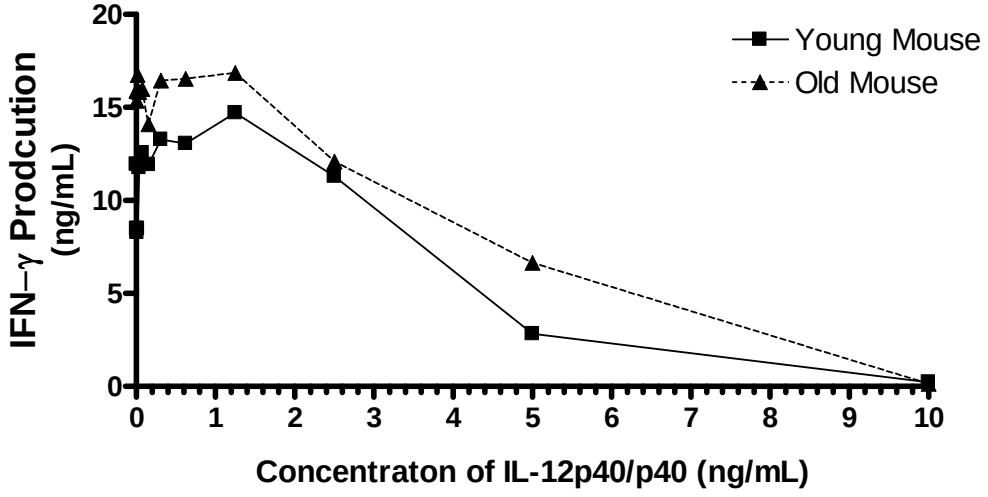

Figure 12. Inhibition by IL-12p40/p40 of IFN–γ production by purified $CD8^+$ T cells from spleen cells of young and old mice. Purified $CD8^+$ T Cells from the spleens of 2-3 or 17-18 month-old mice were cultured for 48 h with media, IL-12p70, IL-18 and IL-12p40/p40. IFN-γ production was analyzed using ELISA. Data is representative of two independent experiments.

Discussion

Tuberculosis is a disease that affects a wide range of people but is most serious in the elderly and immune-compromised patients (4). Due to an increased susceptibility in old age, it is imperative to explore strategies that take advantage of the early transient resistance to tuberculosis seen in mice. An exploration of the mechanism behind this early resistance can lead to more effective treatments in the elderly. A step to understanding this mechanism is characterizing the cytokine responsible for IFN-γ production.

It has been shown that old mice are able to produce IFN-γ in an antigen independent manner (8). Following aerosol infection Th1 cytokines are significantly higher in the lungs of old mice as compared to the lungs of young mice (9). We hypothesized that IL-12p70 is responsible for stimulating $CD8^+$ T cells, which are important in an antigen independent response. This study showed that IL-12p70 is a stimulation factor of antigen independent IFN-γ production. We hypothesized that IL-18 would have a synergistic effect on IL-12p70. Our findings indicated that IL-12p70 acting alone produced low amounts of IFN-γ. But when acting synergistically with IL-18, IL-12p70 is able to produce a large amount of IFN-γ. It was determined that optimal production occurred when IL-12p70 was at a concentration of 5ng/ml and when cultured for 48 hours. IL-12p70 is the biologically active cytokine from the IL-12 family that can stimulate spleen cells to secrete IFN-γ.

This study also showed the IL-12p40 and IL-12p40/p40 have little or no capacity to stimulate IFN-γ production. The level of IL-12p40 mRNA in the lungs of old mice post infection with *M.tb* suggests that IL-12p40 and IL-12p40/p40 maybe present during infection. Despite their possible presence early post infection, this study showed that IL-12p40 and IL-12p40/p40 do not stimulate $CD8^+$ T cells to produce IFN-γ. Our results show that IL-12p40 and IL-12p40/p40 may not be important factors in the early resistance to *M. tb* despite their possible presence in the lung during infection.

This study also investigated IL-23, a close relative of IL-12, consisting of the p40 subunit of IL-12 and a p19 subunit. Our findings indicate that IL-23 does not mediate biological activity of T cells to produce IFN-γ. Though they share a common subunit, our findings showed IL-23 does not to inhibit the action of IL-12p70.

We hypothesized that IL-12p40/p40 would have an inhibitory effect on the action of IL-12p70. We were able to show that IL-12p40/p40 inhibits the action of IL-12p70. IL-12p40 mRNA levels post infection indicate that IL-12p40/p40 maybe present during infection. Western blot analysis is a potential investigatory route to determine whether or not IL-12p40/p40 is actually present. In addition, we showed that IL-12p40 has a small capacity to decrease the amount of IFN-γ produced.

In focusing on CD8+ T cells, our findings in the spleen cell were verified. In the purified CD8+ T cells, IL-12p70 acting with IL-18 was the only IL-12 family member capable of stimulating IFN-γ production. Analogous to the whole spleens cells, IL-12p40/p40 inhibited IL-12p70 from stimulating IFN-γ production by CD8+ T cells.

In some cases, such as the whole spleen IL-12p40/40 inhibition study, it took many experimental repeats to verify results though all results were verified with

repeatable outcomes. The model used in these experiments was non-infectious. Verifying these results using an infectious model would prove valuable to understanding the mechanism behind the early, transient resistance to *M.tb* infection seen in old mice.

In summary, we found that IL-12p70 is the biologically active factor that stimulates IFN-γ. There was no detectable difference between young and old CD8 T cells ability to produce IFN-γ. We also showed the IL-12p40/p40 can inhibit the action of IL-12p70. The next step is to investigate how IL-12p40/p40 affects IL12p70 mediated IFN-γ production *in vivo* and its affect on the early transient resistance. An investigation into the use of IL-12p70 as a therapy to enhance resistance to tuberculosis would be useful as well as looking into the use of IL-12p40/p40 as a method of counter-acting excess IL-12p70 production.

Acknowledgements

I would like to specially thank Dr. Turner and Dr. Vesosky for their time, training of technique and mentorship during the course of this project. I would like to Dr. Turner, Dr. Vesosky, Dr. Beamer and Erin Rottinghaus for their careful reading of this paper, as well as Dr. Assogba for his support everyday in lab. Another thanks to those same people, all of whom assisted me with my day to day activities in the lab and without that help this project would have not been possible.

References

1. Trends in Tuberculosis – United State. Center for Disease Control. 2006
2. Raja, A. 2004. Immunology of tuberculosis. Indian J Med Res 120, 213-232
3. Tuberculosis Fact Sheet. WHO. 2007
4. Tuberculosis (TB): Mycobacteria: Merck Manual Professional. 2005
5. Flynn JL, Chan J. Immunology of Tuberculosis. Annu Rev Immunol. 2001;19:93-129
6. Beers, N.H., R. Berkow, The Merck Manual of Geriatrics, 2006.
7. Turner J, Frank AA, Orme IM. Old mice express a transient early resistance to pulmonary tuberculosis that is mediated by CD8 T cells. Infect Immun. 2002 Aug;70(8):4628-37
8. Vesosky B, Flaherty DK, Rottinghaus EK, Beamer GL, Turner J. Age dependent increase in early resistance of mice to Mycobacterium tuberculosis is associated with an increase in CD8 T cells that are capable of antigen independent IFN-gamma production. Exp Gerontol. 2006 Nov;41(11):1185-94. Epub 2006 Oct6
9. Vesosky B, Flaherty DK, Turner J., Th1 cytokines facilitate CD8-T-cell-mediated early resistance to infection with Mycobacterium tuberculosis in old mice., Infect Immun. 2006 Jun;74(6):3314-24
10. Gubler U, Chua AO, Schoenhaut DS, Dwyer CM, McComas W, Motyka R, Nabavi N, Wolitzky AG, Quinn PM, Familletti PC, et al., Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor., Proc Natl Acad Sci U S A. 1991 May 15;88(10):4143-7
11. Brunda MJ., Interleukin-12., J Leukoc Biol. 1994 Feb;55(2):280-8

12. Carra G, Gerosa F, Trinchieri G., Biosynthesis and posttranslational regulation of human IL-12., J Immunol. 2000 May 1;164(9):4752-61

13. Yoshimoto T, Kojima K, Funakoshi T, Endo Y, Fujita T, Nariuchi H., Molecular cloning and characterization of murine IL-12 genes., J Immunol. 1996 Feb 1;156(3):1082-8

14. Macatonia SE, Hosken NA, Litton M, Vieira P, Hsieh CS, Culpepper JA, Wysocka M, Trinchieri G, Murphy KM, O'Garra A., Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells., J Immunol. 1995 May 15;154(10):5071-9

15. Moller DR, Wysocka M, Greenlee BM, Ma X, Wahl L, Flockhart DA, Trinchieri G, Karp CL., Inhibition of IL-12 production by thalidomide., J Immunol. 1997 Nov 15;159(10):5157-61

16. Hölscher C., The power of combinatorial immunology: IL-12 and IL-12-related dimeric cytokines in infectious diseases., Med Microbiol Immunol. 2004 Feb;193(1):1-17.

17. Sartori A, Ma X, Gri G, Showe L, Benjamin D, Trinchieri G., Interleukin-12: an immunoregulatory cytokine produced by B cells and antigen-presenting cells., Methods. 1997 Jan;11(1):116-27

18. Airoldi I, Gri G, Marshall JD, Corcione A, Facchetti P, Guglielmino R, Trinchieri G, Pistoia V., Expression and function of IL-12 and IL-18 receptors on human tonsillar B cells., J Immunol. 2000 Dec 15;165(12):6880-8

19. Schoenhaut DS, Chua AO, Wolitzky AG, Quinn PM, Dwyer CM, McComas W, Familletti PC, Gately MK, Gubler U., Cloning and expression of murine IL-12., J Immunol. 1992 Jun 1;148(11):3433-4

20. Yao BB, Niu P, Surowy CS, Faltynek CR., Direct interaction of STAT4 with the IL-12 receptor., Arch Biochem Biophys. 1999 Aug 1;368(1):147-55

21. Watford WT, Hissong BD, Bream JH, Kanno Y, Muul L, O'Shea JJ., Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4., Immunol Rev. 2004 Dec;202:139-56

22. Stober D, Schirmbeck R, Reimann J., IL-12/IL-18-dependent IFN-gamma release by murine dendritic cells., J Immunol. 2001 Jul 15;167(2):957-6

23. Okamura H, Kashiwamura S, Tsutsui H, Yoshimoto T, Nakanishi K., Regulation of interferon-gamma production by IL-12 and IL-18., Curr Opin Immunol. 1998 Jun;10(3):259-64.

24. Dinarello CA., Interleukin-18., Methods. 1999 Sep;19(1):121-32

25. Dutt AK. Tuberculosis. Clin Ger Med 1992; 24:36-41